FURTHER EVIDENCE OF THE DESTRUCTION OF BIVALVE LARVAE BY BACTERIA [1]

ROBERT R. L. GUILLARD [2]

The Oyster Institute of North America, Inc.

Loosanoff (1954) has discussed the significance of large-scale culture of larval oysters and hard clams (*Venus mercenaria*) for experimental purposes and potential commercial use. It has been found that various organisms—the fungus *Sirolpidium*, for example—can destroy larvae in such cultures. However, evidence that bacteria are injurious has been largely circumstantial. Davis and Chanley (1956) showed that larval mortality was decreased at times by the use of various antibiotics. Walne (1956, 1958) found that antibiotics brought about increased spatfall of European oysters and parallel decrease of the bacterial population in his culture vessels.

Davis (1950, 1953), as part of feeding experiments, fed fourteen species of bacteria (including a mixture of *B. coli* and a bacteriophage) to oyster larvae. There was no evidence that any bacteria were of value as food. The four species used in the first experiments (*Vibrio marinofulvus, Micrococcus maripuniceus, Bacillus imomarinus,* and a red sulfur bacterium) were harmful at (unspecified) high concentrations, but not at low ones. However, three phytoflagellates studied produced the same results. In the second experiments, larvae fed bacteria (at unspecified concentrations) died within eleven days, while unfed controls grew slightly. Apparently mortality was not catastrophic. In this connection, observations by ZoBell and Feltham (1938) are significant. They found that adult mussels survived and grew when fed 10^5 to 10^9 washed bacterial/ml. once a day for nine months. Thirty-one clones were used. However, when a non-toxic peptone was added to water containing mussels, the animals died when the concentration of bacteria was only of the order of 10^5/ml. They suggested that metabolites in actively growing cultures were responsible, but it is possible that a different flora was selected by the addition of fresh nutrient solution. Brisou (1955) points out that *Pseudomonas*-like organisms are common in living bivalves. Takeuchi *et al.*, (1957) reported a high mortality of adult *Ostrea gigas* caused by or associated with a bacterium of the genus *Achromobacter*.

This report presents evidence that two clones of bacteria isolated from an infected hard clam (*Venus mercenaria*) larva destroyed healthy larvae, while other clones did not under similar experimental conditions. In the final experiments larvae were reared under aseptic conditions up to the time of exposure to known bacteria, thus excluding the possibility that contaminating microorganisms were the direct cause of death, while the bacteria were secondary invaders.

[1] This work was carried out at the Biological Laboratory, U. S. Fish and Wildlife Service, Milford, Connecticut.

[2] Present address: Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.

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Materials and Methods

Isolation and growth of bacteria

Bacteria were seen "swarming" about moribund clam larvae in a laboratory culture having heavy mortality. One larva was transferred to a tube of sea water broth (essentially medium STP of Provasoli *et al.,* 1957), and the resulting mixed flora was subcultured daily. Pour and streak plates were made on the second day. Although most colonies appeared to be of two types, twelve obviously different clones were isolated. Ten other clones were isolated from contaminated algal cultures (*Monochrysis lutheri* or *Isochrysis galbana*) or from filters through which laboratory sea water was passed. Cultures of mixed bacteria were obtained by adding raw sea water to sterile broth.

Bacteria were grown at 28.5° C. for about 24 hours, with resulting concentrations of most clones of the order of 10^9/ml. STP broth was used in some of the first studies, but the medium was later standardized to ⅔ strength (Difco) nutrient broth made with sea water. In Experiment 2(2) bacteria were also grown in a clam broth made by autoclaving a minced adult hard clam in its own volume of sea water and decanting the supernatant. Suspensions of bacteria were diluted and samples killed and stained with I_2-KI and counted in a Petroff-Hausser chamber.

Filtrates of bacterial cultures (Experiment 2(3)) were prepared by drawing a few milliliters through sterile ultra-fine fritted glass filters. Filtrate was proved sterile by plating and inoculation into broth.

Bacteria were killed (Experiment 2(4)) by heating to 85° C. for five minutes. Cultures so heated did not grow upon subculture or streaking .

The concentration of bacteria to be used in the final experiments was estimated from measurement of concentrations in preliminary studies, which were of the order of 10^6/ml., and from observations made at various times during 1957–1958 of concentrations in apparently healthy larval clam and oyster cultures. In plate counts from 24–48-hour larval cultures (made on STP agar, ZoBell's No. 2216 agar (ZoBell, 1941), or ⅔ strength nutrient agar) bacterial concentrations were 10^5–10^6 per ml. However, counts of some of these cultures with a Petroff-Hausser chamber and dark field or phase contrast illumination yielded numbers of motile or clearly recognizable bacteria about an order of magnitude higher. (Concentrations in freshly changed cultures were of the order of 10^3–10^4 per ml., determined by plating.) Walne (1958) measured 10^4–10^5/ml. in 24-hour laboratory cultures of European oysters by plating on ZoBell's No. 2216 agar counted at 48 hours. It may be assumed that the actual concentrations were an order of magnitude higher.

Bacterial concentrations used in the final experiments were 10^6 to 10^7 per ml., provided from dense liquid cultures. Dilution made carryover of nutrients insignificant. (Because larvae had been observed to survive exposure to bacterial concentrations of the order of 10^8 per ml., this also was tried in Experiment 1. It was anticipated that larvae might be resistant to the bacteria because of pretreatment with antibiotics.)

Preliminary assays with non-aseptically-reared larvae

Bacterial cultures were assayed by adding 1- to 4-ml. aliquots yielding 10^6 to 10^7 bacteria per ml. to liter cultures of healthy larvae in freshly changed filtered sea water. (Methods of handling larvae are cited in a later section.) Larvae were maintained at a concentration of *ca.* 10/ml. at 24° C. and fed bacteria-free *Isochrysis galbana, ca.* 5×10^4/ml.

After 18–30 hours larvae were concentrated by screening and swirling and examined in a Sedgwick-Rafter cell with a compound microscope ($\times$ 150). Because the basic purpose of these assays was to screen bacterial cultures for obviously virulent strains, quantitative counts of mortality were not generally made. Rough quantitative counts were easily made by counting fewer than ten fields.

It was shown that the sterile nutrient broths were non-injurious to larvae at the concentrations used. This was done by adding sterile broth plus the antibiotic mixtures described below.

In each experiment there were two sets of control larvae; one received only food, the other received also an aliquot of sterile broth. In a few experiments bacteria developing in this latter beaker during the experimental period destroyed the larvae. These experiments were discarded even though the same flora might not have developed in the assay beakers.

Assay with aseptically-reared larvae

Straight-hinge clam larvae were obtained free of bacteria by allowing fertilized eggs to develop in solutions of antibiotics shown to be harmless to the animals. In the first experiment 50 mg./l. each of penicillin G (1625 units/mg.) and streptomycin sulfate were used. These concentrations were doubled in the second experiment and 50 mg./l. of chloramphenicol added. Oppenheimer (1955) showed that similar mixtures reduced viable bacteria in sea water to the order of a few per ml. in 24 hours. In our experiments, because adult clams were spawned in sterile sea water and larvae isolated with a micropipette after exposure to antibiotics, chances of contamination were negligible. No bacteria were detected by isolating larvae into sea water nutrient broth. Antibiotics carried over in the isolation technique were insufficient to prevent growth of bacteria.

Procedure

Sea water was autoclaved. Spawning dishes and screens were sterilized with ethanol and rinsed. Adult clams of known sex were washed in warm tap water, rinsed, and spawned by methods described or referred to by Davis (1953). Water was changed if clams did not spawn within a few hours. Fertilized eggs were passed through a 100-mesh screen to free them of feces and pseudofeces, then washed on a 325-mesh screen to free them of excess sperm and to concentrate them. After resuspension and counting, about 2000 eggs were added to 100 ml. of sterile filtered antibiotic mixture in 250-ml. Ehrlenmeyer flasks and kept at 24° C. Twenty-four or 36 hours later the fluid was poured into sterile Petri dishes and apparently healthy larvae caught with a pipette under a dissecting microscope.

Thirty to forty larvae were put into each of a series of 20 × 125-mm. screw-capped culture tubes containing 5 ml. of sterile sea water, and fed bacteria-free *Isochrysis galbana* at a concentration of 10^5/ml. (Control larvae in unchanged water in these tubes went to metamorphosis, but more slowly than those kept in containers as described by Davis and Guillard, 1958.) Aliquots of counted bacterial suspensions were added by pipette.

Motility of larvae could be observed through the test tube walls. At the end of the experiments, clams were again poured into a Petri dish and re-isolated onto a slide, where each was examined with a compound microscope using dark field and phase contrast illumination as necessary.

Experimental

Assay with non-aseptically-reared larvae

Mortality of larvae exposed for 18–30 hours to the mixed culture derived from a moribund clam and to the five succeeding subcultures from it was greater than 90%. "Swarming" bacteria were numerous in moribund animals and were seen swimming freely. No comparable mortality was observed in five trials involving the ten clones derived from contaminated algal cultures or the mixed bacteria resulting from the enrichment of sea water. (Not all clones were included in each of the trials.) Larvae were able to survive in concentrations as high as 10^8/ml. of some of these clones, though they developed abnormally in the higher concentrations. Mortality was negligible in both sets of controls in all these trials.

Two assays were made of the twelve clones isolated from the moribund clam. Ten of these caused no significant mortality, but the other two produced mortality comparable to that of the mixture from which they were derived. These clones, designated 6–1 and 13–1, were the two colony types predominating on agar plates of the mixed culture. Both are gram-negative, non-sporogenous, polar monotrichous rods about 0.75 × 1–2.5 microns in size. Both are halophilic to some extent and have not become adapted to growth on media without NaCl or sea water. The temperature optimum of 6–1 is between 35° and 40° C., while that of 13–1 lies between 25° and 32.5° C. Dr. Einar Liefson of Loyola University has undertaken further study of both strains. He has assigned 6–1 to the genus *Vibrio* and 13–1 to *Pseudomonas*. Some criteria are given in Hugh and Leifson (1953).

Experiment showed that neither 6–1, 13–1, nor the mixture from which they came could injure larvae in the presence of antibiotics. Duplicate larval cultures were inoculated with *ca.* 10^7 bacteria per ml.; to one set was added also 50 mg./l. each of penicillin G and streptomycin sulfate. Animals exposed to bacteria alone were destroyed in 24 hours, while those treated with antibiotics also were indistinguishable from controls exposed to neither or to antibiotics alone. This was done twice with each bacterial culture.

Experiments with aseptically-reared larvae

Experiment 1. This was undertaken primarily to test the method and to determine if bacteria at the concentrations used would kill larvae previously exposed to antibiotics. The experiment proper consisted of eight tubes, as follows:

1. controls, sea water
2. controls, nutrient broth
3. clone 13–1, 10^6/ml.
4. clone 13–1, 10^7/ml.
5. clone 13–1, 10^8/ml.
6. clone 6–1, 10^6/ml.
7. clone 6–1, 10^7/ml.
8. clone 6–1, 10^8/ml.

There were also five tubes in which larvae were grown to metamorphosis.

Examination through the tube walls showed that most larvae were killed in a day in the highest concentration of each clone. The other tubes were examined on the fourth day. Results are summarized in Table I. Larvae exposed to 10^7/ml.

TABLE I

Mortality of clam larvae caused by three concentrations of clones 6–1 and 13–1 (larvae initially 115–120 microns in size)

Clone	Bacteria per ml.	Time	No of larvae moribund	No. of larvae recovered	% of larvae moribund	Notes
13–1	10^8	1 day	26	27 of 30	96	vela disintegrating
13–1	10^6	4 days	3	29 of 30	10	none over 135 μ
13–1	10^7	4 days	38	40 of 40	95	none over 135 μ
6–1	10^8	1 day	27	32 of 35	84	none over 125 μ
6–1	10^6	4 days	22	29 of 30	76	largest were 160 μ; dead larvae were 120–145 μ
6–1	10^7	4 days	11	11 of ?	100	vela swollen; none over 135 μ
Control	none	4 days	0	30	0	135–160 μ*

* Another group of larvae from the same spawning was kept in polyethylene containers, fed every day and changed every other day. The largest of these larvae were 190 μ.

of either clone were 95%–100% destroyed by the fourth day. However, 10^6/ml. of 13–1 caused only 10% mortality, while a like number of 6–1 killed 76%. No control animals died.

Bacteria were not counted during or after the experiment. Judging subjectively, there were not as many in the medium at the end as might have expected on the basis of the number added. Some of the moribund larvae were surrounded by "swarming" bacteria.

Experiment 2. The objectives were:

1. To compare the effects of bacteria on larvae kept at three different temperatures; 20, 25, and 30° C. The observation that clones 6–1 and 13–1 grew better at high temperatures than did most other clones isolated suggested this portion of the experiment.

2. To compare the effects on larvae of equal numbers of bacteria grown on two different media—the usual nutrient broth and clam broth. The possibility that bacteria maintained on clam broth might remain more virulent than those maintained on nutrient broth prompted this portion of the experiments.

3. To observe larvae exposed to sterile filtrate of bacterial cultures.

4. To observe larvae exposed to dead bacteria plus their culture broth. Experiments 3 and 4 were to confirm that only living bacteria kill larvae, as suggested by the antibiotic experiment of Section 1. The experiment was carried out in 20 tubes, as follows:

Tube	Temperature ° C.
1. Control, food only	25
2. 6–1, 10^7/ml. (broth-grown)	25
3. 13–1, 10^7/ml. (broth-grown)	25
4. Control, food only	20
5. 6–1, 10^7/ml. (broth-grown)	20
6. 13–1, 10^7/ml. (broth-grown)	20
7. Control, food only	30
8. 6–1, 10^7/ml. (broth-grown)	30
9. 13–1, 10^7/ml. (broth-grown)	30
10. 6–1, grown in clam broth, 10^6/ml.	25
11. 6–1, grown in clam broth, 10^7/ml.	25
12. 13–1, grown in clam broth, 10^6/ml.	25
13. 13–1, grown in clam broth, 10^7/ml.	25
14. 6–1 filtrate, 1 ml. (equivalent to 1.6×10^9 bacteria/ml.)	25
15. 13–1 filtrate, 1 ml. (equivalent to 3.2×10^9 bacteria/ml.)	25
16. Heat-killed 6–1 (8×10^8/ml.)	25
17. Heat-killed 13–1 (8×10^8/ml.)	25
18. Larvae in broth alone as sterility check	25
19. Larvae in broth alone as sterility check	25
20. Larvae in broth alone as sterility check	25

Larvae were examined on the fifth day. In Table II and Table III, respectively, are gathered data pertinent to the temperature portion of the experiment and comparison of the effects of bacteria grown on different media.

Mortality was relatively independent of the temperature at which larvae were kept: 73% to 76% of the animals exposed to clone 6–1 were dead, as were 30% to 48% of those exposed to clone 13–1. Maximum mortality in controls was 7%. It should be noted that growth in the 30° C. controls was poor and that food organisms settled in the tubes at this temperature. (Both 20° and 30° controls were bacteria-free at the end of the experiment.)

From Tables I and II it can be seen that there were no consistent differences in mortality caused by bacteria grown on the two different media. Greatly increased virulence would have been evidenced by early heavy mortality easily visible through the culture tube walls. Small but significant differences would not be detected by an experiment such as this.

The hypothesis that only living bacteria destroy larvae was confirmed by the findings in tubes 14 through 17, in which larvae were exposed to filtrate corresponding to more than 10^9 bacteria per ml. or to 8×10^8 dead bacteria in their broth. Of 120 larvae, only two were dead, one in tube No. 15 and one in No. 17. How-

TABLE II

Comparison of mortality of larvae exposed to bacteria and maintained at three different temperatures for five days (concentration of bacteria 10^7/ml. Larvae initially 105–110 microns in size)

Clone	Temp. ° C.	% larvae moribund	Average size		Maximum size	
			Living	Moribund	Living	Moribund
6–1	20	73	125	120	145	125
6–1	25	75	130	115	150	125
6–1	30	76	120	120	130	120
13–1	20	30	150	125	165	150
13–1	25	46	145	125	160	140
13–1	30	48	135	120	185	135
None	20	7	145		165	—
None	25	0	140		165	—
None	30	3	125	—	140	—

ever, larvae grew scarcely at all. They were initially 105–110 microns in size, and increased only to 110–120 microns. (The larger animals seen in control tubes were 165 microns in size, see Table III.) Moreover, larvae exposed to filtrate or dead bacteria were not feeding and were emaciated. This was probably due to the high concentration of metabolites. In the preliminary assays (Section 1) in which larvae were exposed to living bacteria together with antibiotics, those animals exposed to both bacteria and antibiotics were indistinguishable from controls, but the amount of inoculum in this case corresponded only to 10^6–10^7 bacteria/ml. rather than to 10^9/ml.

In both experiments 1 and 2, moribund larvae injured by clone 6–1 differed in appearance from those injured by 13–1. The former often had vela so distended that they exceeded the bodies of the larvae in length. Some vela appeared to be decomposing and occasionally they detached from the rest of the clam. The bodies of larvae often had striations visible going from the hinge towards the velum.

Clams injured by 13–1 usually had vela that were frayed or ragged, with cilia largely or entirely missing. The bodies were emaciated and granular in appearance. Some seemed to be disintegrating from the hinge side towards the velum. Mori-

TABLE III

Comparison of the mortality at 5 days of larvae exposed to bacteria grown in (a) 2/3 strength nutrient broth and (b) clam broth (larvae initially 105–110 microns in size)

Clone	Medium	Bacteria per ml.	% larvae moribund	Average size		Maximum size	
				Alive	Moribund	Alive	Moribund
6–1	nutrient	10^7	75	130	115	150	125
6–1	clam	10^6	40	135	125	150	135
6–1	clam	10^7	97	120	120	120	120
13–1	nutrient	10^7	46	145	125	160	140
13–1	clam	10^6	59	135	120	140	135
13–1	clam	10^7	48	135	120	150	135
Controls		none	none	140	—	165	—

bund larvae in laboratory cultures often fit one of these descriptions, but it is not known if the appearance is in fact correlated with an infecting bacterium.

Discussion

While the mechanism by which clones 6–1 and 13–1 destroy larvae was not studied, evidence available favors the hypothesis that it is by invasion or at least contact rather than by an exotoxin liberated into the medium. The fact that larvae withstood relatively large amounts of glass-filtered, heat-killed, or antibiotic-treated bacterial culture shows that an exotoxin, to be the sole agent, would have also to be extraordinarily unstable. Further, an exotoxin would be expected to have a relatively uniform influence on animals exposed to it, so that larvae would be more or less uniform in size. In fact, however, larvae exposed to bacteria varied considerably in size (Tables II and III), rather in keeping with the hypothesis that they continued to grow until invaded. Finally, there are observations that mortality in large cultures often followed the pattern of an epizootic, and that bacteria were frequently seen swarming in dying or dead larvae.

It is not implied that bacterial metabolites are without influence on larval growth or development. Indeed, the experiment showed that high concentrations stopped growth entirely. It has also been observed that bacterial contamination of algal food cultures sometimes caused abrupt decrease in larval growth rate without immediate extensive mortality. The addition of cultures of bacteria (other than 6–1 or 13–1) often did the same. This depressant effect may well be due to exotoxins.

Strains 13–1 and 6–1 were far more virulent than other bacteria tested and clearly are a hazard to larvae under laboratory conditions. Possibly they were favored by conditions in the larval cultures and finally dominated the flora, at which point the "disease" became obvious. The observation that both strains grow well at temperatures over 30° C., which is relatively uncommon in marine bacteria found locally, supports this idea. At present it is not possible to tell if these bacteria destroy larvae in nature, where both bacteria and larvae are usually less concentrated than they are in cultures. If the "disease" occurs in nature, one would expect to find it under conditions of high temperature and restricted water exchange.

It should be mentioned that the use of antibiotics to control bacteria in larval cultures is apparently more effective when the water supply is changed regularly and two or more antibiotics are used alternately. Probably this prevents the development of a resistant flora. Animals other than bivalves are also benefited; J. Hanks (personal communication) found that larvae of the gastropods *Polinices duplicata* and *P. heros* grew better when penicillin and streptomycin were used in this way. It must be emphasized, however, that the same antibiotics will not prevent growth of injurious bacteria in algal cultures used as food. If impure algal cultures must be used to raise larvae, the algae should obviously be kept at the lowest temperature allowing reasonable growth.

Summary

1. Twelve strains of bacteria were isolated from a moribund *Venus mercenaria* larva in a laboratory culture. These, ten other clones, and mixed bacteria from

sea water were assayed by adding broth culture yielding 10^6–10^7 cells/ml. to beaker cultures of healthy clam larvae. Only the mixed bacterial culture from the moribund larva and two of the 12 strains isolated from it caused extensive mortality. One of the virulent clones (6–1) is a species of *Vibrio*, the other (13–1) is a *Pseudomonas* species.

2. Larvae exposed to virulent bacteria and simultaneously treated with antibiotics were as healthy as controls, showing that active bacteria were necessary to destroy larvae and that metabolites in the bacterial inoculum were not harmful to larvae.

3. Larvae were grown free of contaminating micro-organisms by allowing washed eggs to develop in antibiotic solutions and then isolating straight-hinge larvae by pipette. Either virulent clone (10^6–10^7/ml.) destroyed 10–100% of such larvae. However, exposing the animals to large amounts of glass-filtered or heated broth in which bacteria had been grown (corresponding to *ca.* 10^9 bacteria/ml.) caused no mortality, but retarded growth.

4. Mortality caused by clones 6–1 and 13–1 in groups of clams kept at 20°, 25°, and 30° C. did not vary significantly. However, both virulent clones grow well at 30° C. and higher; thus high temperatures in laboratory larval cultures favor these strains.

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